K^+/NH_4^+ antiporter: a unique ammonium carrying transporter in the kidney inner medulla

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Abstract

The mechanism of NH_4^+ transport in inner medulla is not known. The purpose of these experiments was to study the process that is involved in ammonium (NH_4^+) transport in cultured inner medullary collecting duct (mIMCD-3) cells. Cells grown on coverslips were exposed to NH_4^+ and monitored for pH_i changes by the use of the pH-sensitive dye BCECF. The rate of cell acidification following the initial cell alkalinization was measured as an index of NH_4^+ transport. The rate of NH_4^+ transport was the same in the presence or absence of sodium in the media (0.052 ± 0.003 vs 0.048 ± 0.004 pH/min, $P > 0.05$), indicating that NH_4^+ entry into the cells was independent of sodium. The presence of ouabain, bumetanide, amiloride, barium, or 4,4′-di-isothiocyanostilbene-2-2′-disulfonic acid (DIDS) did not block the NH_4^+-induced cell acidification, indicating lack of involvement of Na^+:K^+-ATPase, Na^+:K^+:2 Cl^- transport, Na^+:H^+ exchange, K^+ channel, or Cl^-/base exchange, respectively, in NH_4^+ transport. The NH_4^+-induced cell acidification was significantly inhibited in the presence of high external [K^+] as compared to low external [K^+] (0.018 ± 0.001 vs. 0.049 ± 0.003 pH/min for 140 mM K^+ vs. 1.8 mM K^+ in the media, respectively, $P < 0.001$). Inducing K^+ efflux by imposing an outward K^+ gradient caused intracellular acidification by ~ 0.3 pH unit in the presence but not the absence of NH_4^+. This K^+ efflux-induced NH_4^+ entry increased by extracellular NH_4^+ in a saturable manner with a Km of ~ 5 mM, blocked by increasing extracellular K^+ and was not inhibited by barium. The K^+ efflux-coupled NH_4^+ entry was electroneutral as monitored by the use of cell membrane potential probe 3,3′-dipropylthiadicarbocyanine. These results are consistent with the exchange of internal K^+ with external NH_4^+ in a 1:1 ratio. The K^+-NH_4^+ antiporter was inhibited by verapamil and Schering 28080 in a dose-dependent manner, was able to work in reverse mode, and did not show any affinity for H^+ as a substrate, indicating that it is distinct from other NH_4^+-carrying transporters.

We conclude that a unique transporter, a potassium-ammonium (K^+/NH_4^+) antiport, is responsible for NH_4^+ transport in renal inner medullary collecting duct cells. This antiporter is sensitive to verapamil and Schering 28080, is electroneutral, and is selective for NH_4^+ and K^+ as substrates. The K^+/NH_4^+ antiporter may play a significant role in acid-base regulation by excretion of ammonium and elimination of acid.

Keywords: Inner medulla; NH_4^+; K^+; Antiporter; Acid-base

1. Introduction

Excretion of NH_4^+ by the kidney is essential to acid-base regulation in mammalian species [1]. Stud-

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ies in nephron segments have demonstrated that NH_4^+ is secreted into the lumen of proximal tubule [1,2], reabsorbed in the medullary thick ascending limb [1,3], and transported to the medullary collecting duct cells where it is secreted into the lumen down a favorable NH_3 concentration gradient [4–6]. Microperfusion and influx experiments have illustrated that secretion of newly-synthesized NH_4^+ into the lumen of proximal tubule (PT) is predominantly mediated via Na^+/H^+ exchanger [7,8]. The reabsorption of NH_4^+ in the lumen of medullary thick ascending limb of Henle (mTAL) occurs mostly via Na^+-K^+-$2Cl^-$ cotransport [9–12]. While it has been shown that inner medullary collecting duct (IMCD) is the major segment for secretion of NH_4^+ [1,4–6], the cellular mechanism mediating the transport of NH_4^+ from the interstitium to the medullary collecting duct cells remains unknown. Insight into the cellular mechanism of NH_4^+ transport in this nephron segment would likely increase our understanding regarding a variety of pathologic conditions associated with acid-base abnormalities.

Because of similarities between K^+ and NH_4^+ structure [4], NH_4^+ may substitute for potassium in several transport processes [9–13]. Amongst these transporters, Na^+:K^+:$2Cl^-$ cotransport and Na^+:K^+-ATPase are two known examples [9–13]. Studies in rat kidney have shown that the rate of NH_4^+ reabsorption in the mTAL lumen is affected by K^+ concentration, indicating competition of NH_4^+ and K^+ for the same binding site in luminal Na^+:K^+:$2Cl^-$ cotransporter [9,10]. The interaction of NH_4^+ with the Na^+:K^+-ATPase is less well documented: NH_4^+ has been shown to have affinity for the Na^+:K^+-ATPase in rabbit proximal tubules [14] but not in the opossum kidney cells [15]. In rat IMCD cells [13] or mouse IMCD cells [16] NH_4^+ can decrease ouabain-sensitive or bumetanide-sensitive ^{86}Rb influx, suggesting interaction of NH_4^+ with the K^+ site of Na^+:K^+-ATPase or basolateral Na^+:K^+:2Cl transporter, respectively. These studies [13,16], however, did not measure NH_4^+ transport. As such, the contribution of Na^+:K^+-ATPase or basolateral Na^+-K^+-$2Cl^-$ transporter to total NH_4^+ transport in rat or mouse IMCD cells was not determined. NH_4^+ can also compete with K^+ binding site on K^+ channel [15,17]. Recent studies in cell suspensions from thick ascending limb of rat kidney showed that NH_4^+ could be transported on K^+/H^+ antiport [18] and K^+:Cl^- cotransport [12].

In the present work, we studied NH_4^+ transport in cultured mouse IMCD cells by monitoring pH_i. Cells were exposed to varying NH_4^+ concentrations and the rate of cell acidification following the initial cell alkalinization was then measured as an index of NH_4^+ transport. This cell acidification was only observed in the presence of NH_4^+ and was not mediated via known transporters that interact with NH_4^+. The results provide evidence for the presence of a new transporter, called here K^+/NH_4^+ antiporter, which is responsible for majority of NH_4^+ transport in mIMCD-3 cells. This transporter exchanges intracellular K^+ with extracellular NH_4^+ and is likely responsible for the transport of NH_4^+ from the interstitium to the cells of inner medullary collecting duct. The K^+/NH_4^+ antiport may play an essential role in NH_4^+ excretion into the urine and thus regulation of acid base.

2. Materials and methods

2.1. Cell culture procedures

mIMCD-3 cells were cultured in a 1:1 mixture of Ham's F-12 and Dulbeco's modified Eagle's medium (DME) containing 100 U/ml penicillin-G and supplemented with 10% fetal bovine serum as described [19]. The mIMCD-3 cell line which has been developed from simian virus transgenic mice retains many characteristics of this nephron segment [20]. Cultured cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. The medium was replaced every other day.

2.2. Intracellular pH measurement

Changes in intracellular pH (pH_i) were monitored using the acetoxymethyl ester of the pH-sensitive fluorescent dye 2′,7′bis (carboxyethyl)-5(6)-carboxyfluorescein (BCECF-AM) as described [18,19,21,22]. mIMCD-3 cells were grown to confluence on coverslip and incubated in the presence of 5 μM BCECF in a solution consisting of 140 mM NaCl, 0.8 mM K_2HPO_4, 0.2 mM KH_2PO_4, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Hepes (*N*-2-hydroxyethylouoera-

Table 1
Composition of experimental solutions

Compound	Solutions			
	A	B	C	D
NaCl	140			
KCl			140	1.8
TMA-Cl		140		125
K_2HPO_4	0.8	0.8	0.8	
KH_2PO_4	0.2	0.2	0.2	
$CaCl_2$	1	1	1	1
$MgCl_2$	1	1	1	1
Hepes	10	10	10	10
Glucose	5	5	5	5
Barium				10

Concentrations are in mM. All solutions were bubbled with 100% O_2 and adjusted to pH 7.40.

zube-*N*′-2-ethanesulfonic acid) and 5 mM glucose (solution A, Table 1). To measure the intracellular pH, each coverslip was positioned diagonally in a cuvette and the latter was then placed in a thermostatically controlled holding chamber (37°C) in a Delta Scan dual excitation spectrofluoremeter (PTI, double-beam fluorometer, Photon Technology International, Brunswick, NJ). The monolayer was then perfused with the appropriate solution (Table 1). The perfusion was achieved using a Harvard constant infusion pump. Where indicated, inhibitors or their vehicles were added to the experimental solution in a 1:1000 dilution from a stock solution. The fluorescence ratio at excitation wavelengths of 500 and 450 nm was utilized to determine intracellular pH values in the experimental groups by comparison to the calibration curve. The emission wavelength was recorded at 525 nm. The calibration curve was generated using KCl/nigericin technique and solutions of varying pH. The experiments were performed in isoosmotic CO_2-free media buffered with Hepes. To measure the rate of NH_4^+ transport, cells were exposed to an NH_4^+-containing solution. The rate of cell acidification (dpH_i/dt) following the initial alkalinization was measured as an index of NH_4^+ flux into the cells; this is an established method used by several investigators to determine the rate of NH_4^+ transport into cells of various tissues [12,18]. Initial rates of cell acidification (dpH_i/dt) were estimated from the slopes of pH_i vs. time (t) and were expressed as pH units per minutes. Correlation coefficients for these linear fits averaged 0.976 ± 0.004.

2.3. Preparation of mIMCD-3 cell suspension

mIMCD-3 cells were grown to confluence in 75 cm^2 flasks, rinsed with a calcium- and magnesium-free salt solution and gently rocked for $\sim$30 seconds. Thereafter, the solution was replaced with a cell dissociation solution consisting of phosphate buffered saline (PBS), EDTA, glycerol, and sodium citrate (from Sigma). This solution contains no protein and allows dislodging of cells without the use of enzymes. The cells were incubated in this solution for 5–10 min at 37°C and harvested by pipetting [23].

2.4. Determination of variation in membrane potential

Variation in cell membrane potential was determined with the use of DiS-C3-(5) according to established methods [23,24]. Briefly, the fluorescence of 1 μM DiS-C3-(5) was monitored at excitation and emission wavelengths of 620 and 660 nm, respectively in low or high K^+ solution (Solutions B and C, Table 1) in the absence of cultured cells. The mIMCD-3 cells in suspension were then added to the solution and the quenching of the fluorescence was determined within 30 sec. To demonstrate the validity of this approach, a suspension of cultured mIMCD-3 cells was incubated in high K^+ media to achieve a K^+-loaded state (Solution C, Table 1). The K^+-loaded cells were then diluted into a low K^+ solution (Solution B, Table 1) in the presence of DiS-C3-(5). Thereafter, 5 μM valinomycin was added to the cuvette and the extracellular $[K^+]$ was incrementally increased using aliquots of 3 M KCl (see Section 3). The validity of measurement of membrane potential variation by this method in renal cells has previously been established [18,23–26].

2.5. Materials

F-12/DME medium was purchased from GIBCO-BRL. DiS-C3-(5), BCECF, and nigericin were from Molecular Probes. Valinomycin and quinidine were from Fluka Chemika-Biochemika Analytika.

Amiloride, barium, DIDS, bumetanide, ouabain, and verapamil were purchased from Sigma Chemical Co. Schering 28080 was a generous gift from Schering Corp (via Dr. Cuppoletti, University of Cincinnati, USA).

2.6. Statistics

Results are expressed as means ± S.E. Statistical significance between experimental groups was assessed by Student's *t* test or by one-way analysis of variance.

3. Results

3.1. Ammonium transport in mIMCD-3 cells

To examine whether NH_4^+ enters the mIMCD-3 cells via a carrier-mediated process, cells were grown to confluence on coverslips and monitored for pH_i changes in NaCl solution (solution A, Table 1) or upon exposure to NH_4^+-containing solution (20 mM NaCl was replaced with 20 mM NH_4Cl). Addition of 20 mM NH_4^+ induced a rapid initial cell alkalinization, likely due to NH_3 diffusion (Fig. 1a). Following this initial alkalinization, pH_i decreased to baseline in approx. 10 min. To determine whether this acidification represented NH_4^+ entry, cells were alkalinized to the same level using sodium acetate or sodium propionate withdrawal [13] and monitored for pH_i recovery. As shown in Fig. 1a, except for a small drop, the cell pH did not recover from the initial cell alkalinization during the 10 min of pH_i monitoring. The rate of cell acidification was significantly higher in the presence of NH_4^+ compared to acetate or propionate withdrawal (0.056 ± 0.003 for NH_4^+ vs 0.009

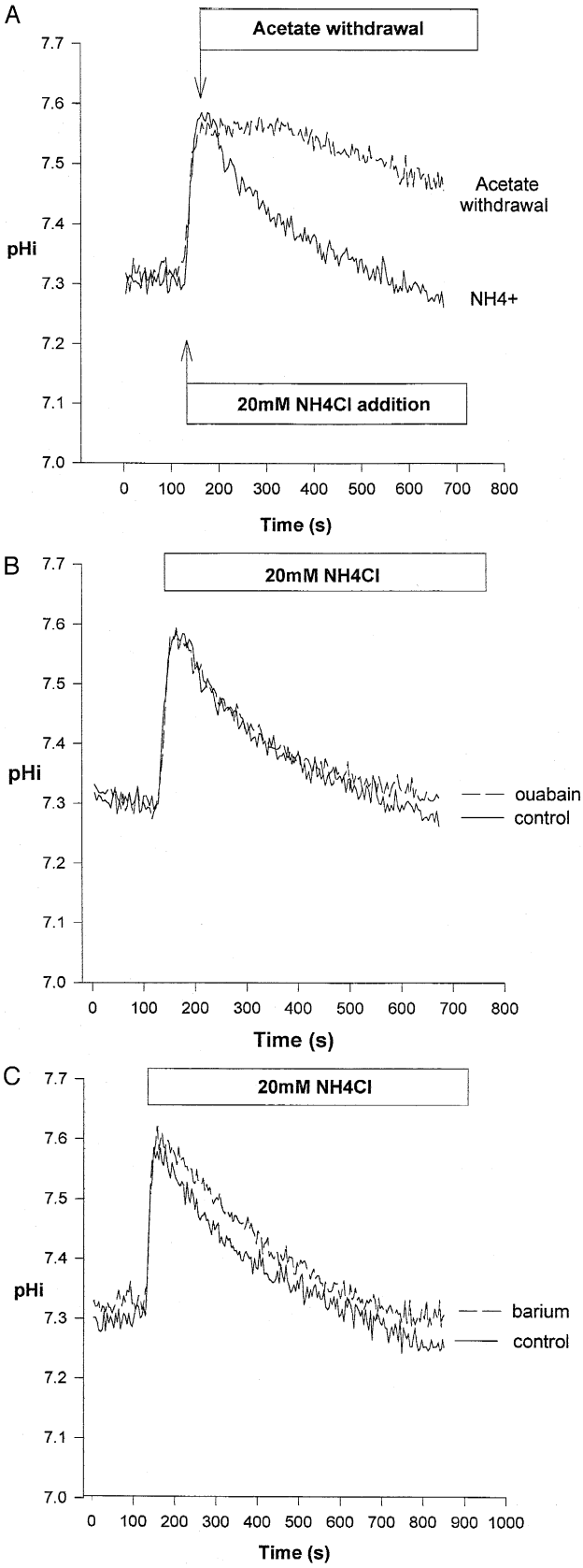


Fig. 1. NH_4^+ transport in mIMCD-3 cells. (a) mIMCD-3 cells were alkalinized by preincubation and withdrawal of 20 mM acetate ($n = 5$) or were exposed to 20 mM NH_4Cl ($n = 5$). (b and c) Representative tracings showing exposure of mIMCD-3 cells to 20 mM NH4Cl in the presence of 1 mM ouabain ((b), $n = 6$) or 10 mM $BaCl_2$ ((c), $n = 5$) as compared to control (no inhibitor). Barium experiments were performed in phosphate-free solution (solution D, Table 1) in order to prevent Ba-phosphate precipitation. All inhibitors or their vehicles were added to the cells 2 min before NH_4^+ addition.

± 0.002 for acetate withdrawal, $P < 0.0001$, and 0.007 ± 0.002 pH/min for propionate withdrawal, $P < 0.0001$). There was no difference in peak pH_i values acheived in NH_4Cl addition (7.567 ± 0.008) and acetate (7.557 ± 0.009, $P > 0.4$; Fig. 1a) or proionate (7.549 ± 0.011, $P > 0.2$; $n = 4$, data not shown) withdrawal. These results indicate that recovery from alkaline pH_i in the presence of NH_4^+ in mIMCD-3 cells is due to NH_4^+ entry.

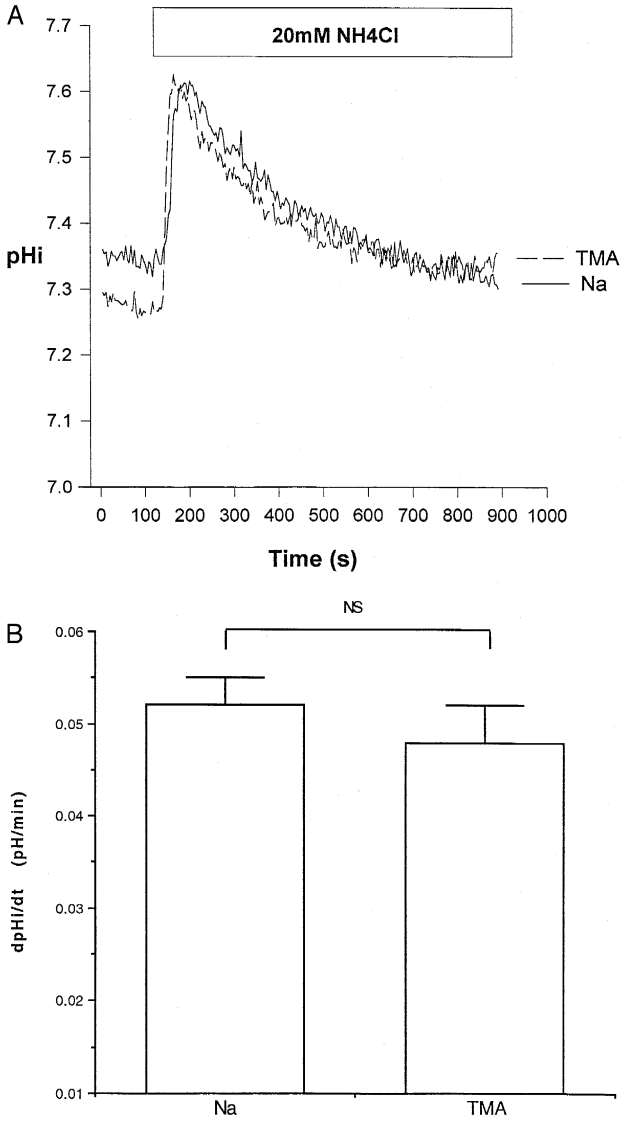


Fig. 2. Effect of Na^+ on NH_4^+ transport. mIMCD-3 cells were incubated in Na^+-containing (solution A, Table 1) or Na^+-free solution (solution B, Table 1) and assayed for NH_4^+ transport. a. Representative tracing demonstrating NH_4^+ transport in the presence or absence of sodium. Each datum (b) represents the mean ± S.E. for the initial rate of NH_4^+-induced cell acidification (dpH_i/dt) of indicated n runs.

Table 2
Effect of amiloride, barium, bumetanide, DIDS, and ouabain, on $dpHi/dt$ induced by NH_4^+ entry into the cells

Groups	(n)	dpH_i/dt (pH U/min)	P
Vehicle	5	0.047 ± 0.006	
Amiloride	5	0.049 ± 0.007	NS
Control	4	0.049 ± 0.005	
Barium	5	0.042 ± 0.003	NS
Vehicle	4	0.048 ± 0.004	
Bumetanide	6	0.045 ± 0.002	NS
Vehicle	4	0.050 ± 0.006	
DIDS	5	0.047 ± 0.003	NS
Vehicle	4	0.054 ± 0.003	
Ouabain	6	0.060 ± 0.004	NS

Values are measn ± SE; n, number of coverslip; dpH_i/dt, the rate of cells acidification induced by NH_4^+ entry into the cells. mIMCD-3 cells were incubated in Na^+-containing solution (Solution A, Table 1), in the absence (vehicle, DMSO) or presence of 500 μM amiloride, 500 μM bumetanide, 100 μM DIDS (4,4′-di-isothiocyanostilbene-2-2′-disulfonic acid) or 1 mM ouabain 2 min. before NH_4Cl addition. 10 mM barium ($BaCl_2$) was added isoosmoticaly to the solution A (15 mM NaCl replaced with 10 mM $BaCl_2$), in the absence of phosphate ($KHPO_4$ replaced with 1.8 mM KCl).

3.2. Mechanism of NH_4^+ transport in mIMCD-3 cells

The mIMCD-3 cells express a Ba^{++}-sensitive K^+ channel and an amiloride-sensitive Na^+ channel [20] on their apical membranes, and a ouabain-sensitive Na^+-K^+ATPase, a furosemide-sensitive Na^+-K^+-$2Cl^-$ cotransporters [27] and two isoforms of Na^+/H^+ exchanger (NHE-1 and NHE-2) on their basolateral membranes [19]. Since NH_4^+ has been shown to be transported via these pathways in other tissues [1,7–12], we sought to determine whether NH_4^+ entry into mIMCD-3 cells was mediated by any of these transporters.

Cells were incubated in Na-containing media (solution A, Table 1) and then exposed to 20 mM NH_4Cl in the presence of ouabain at 1 mM (Fig. 1b) or barium at 10 mM (Fig. 1c). In addition to ouabain and barium, the effect of amiloride, 500 μM, bumetanide, 500 μM, or DIDS, 100 μM, on NH_4^+ transport was also tested. The summary of the results is included in Table 2 and shows that none of these

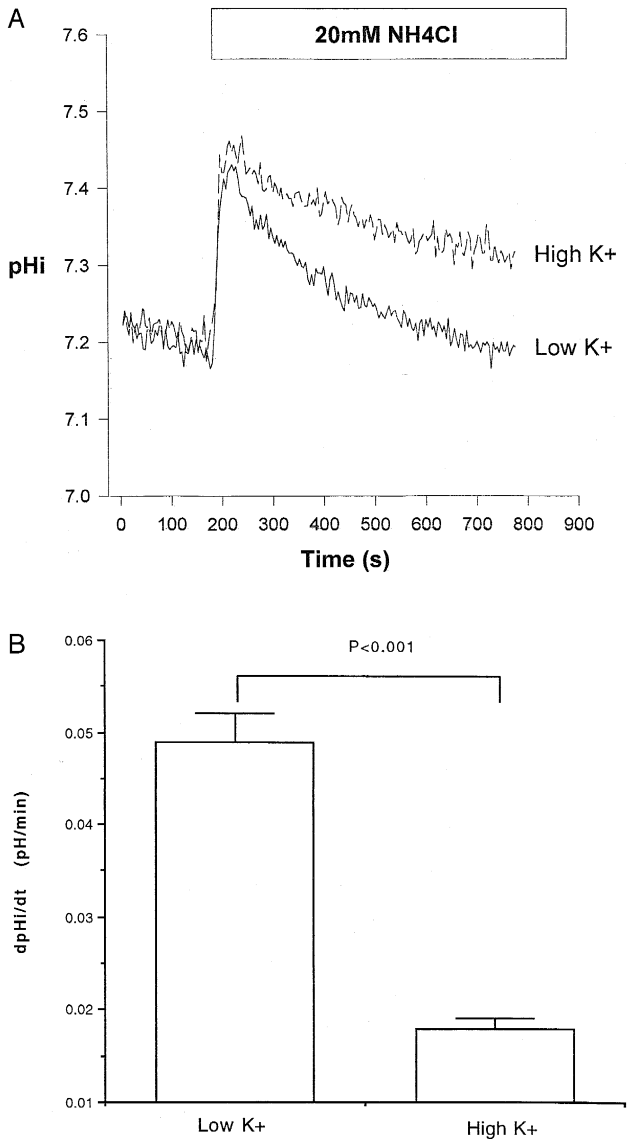


Fig. 3. Effect of high and low K^+ on NH_4^+ transport. Cells were incubated in Na-free solution in the presence of low or high K^+ (solutions B and C, Table 1) and assayed for for NH_4^+ transport. a. Representative tracing demonstrating NH_4^+ transport in the presence of high or low K^+. Each datum (b) represents the mean ± S.E. for the initial rate of NH_4^+-induced cell acidification (dpH_i/dt) of indicated *n* runs for high or low K^+.

inhibitors altered pH_i recovery as determined by dpH_i/dt. These results indicate that the corresponding transporters for these inhibitors (Na^+:K^+-ATPase, K^+ channel, Na^+/H^+ exchange or sodium channel, Na^+:K^+:$2Cl^-$ cotransport, and Cl^-/base exchange) do not mediate the transport of NH_4^+ in mIMCD-3 cells. In additional experiments, we found that presence or absence of extracellular calcium had no effect on NH_4^+ entry into mIMCD-3 cells (0.042 ± 0.008 in

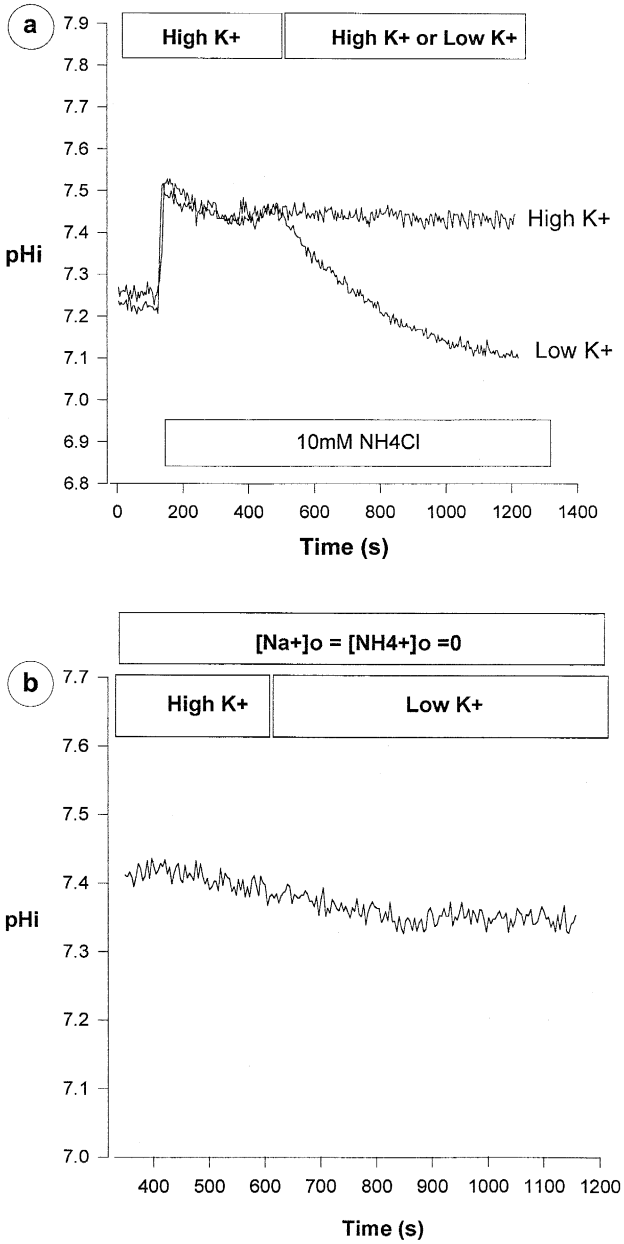


Fig. 4. K^+ efflux-induced NH_4^+-dependent cell acidification. mIMCD-3 cells were incubated in a Na^+-free high K^+ solution (solution C, Table 1) and then exposed to 10 mM NH_4Cl. At steady state ($pH_i \sim 7.40$), cells were perfused with either the same solution or switched to a Na^+-free low K^+ solution that contained 10 mM NH_4Cl. Representative tracings show the effect of an outward K^+ gradient imposition on cell pH as compared to no outward K^+ gradient in the presence (a) or absence of NH_4^+ (b).

the presence and 0.039 ± 0.003 pH/min in the absence of Ca^{2+}, $P > 0.05$).

To determine whether NH_4^+ transport in mIMCD-3 cells occurs via a Na-dependent pathway, NaCl was replaced with equimolar concentration of TMACl (tetramethylammonium chloride) (solution B, Table 1). Fig. 2a shows representative pH_i tracings of NH_4^+ entry in mIMCD-3 cells in the presence or absence of sodium. As further shown in Fig. 2b, the rate of NH_4^+ entry (NH_4^+-induced cell acidification) was not altered in the presence of sodium in the media (0.052 ± 0.003 in the presence of Na^+ vs 0.048 ± 0.004 pH/min in the absence of Na^+, $P > 0.05$), indicating that the transport of NH_4^+ into mIMCD-3 cells was sodium independent. The rest of the experiments (Figs. 3–9) were performed in Na-free solution.

3.3. Interaction of external K^+ with NH_4^+ transport

It has been shown that the hydrated radius of NH_4^+ is very similar to that of K^+ and, therefore, may compete with K^+ for the same binding site [4]. To determine whether NH_4^+ transport in mIMCD-3 cells interacts with K^+, cells were incubated in the presence of low K^+ or high K^+ solution (Table 1, solutions B and C) and monitored for pH_i changes upon exposure to ammonium (20 mM TMA-Cl or KCl was replaced with 20 mM NH_4Cl). Representative pH_i tracings (Fig. 3a) demonstrate that NH_4^+

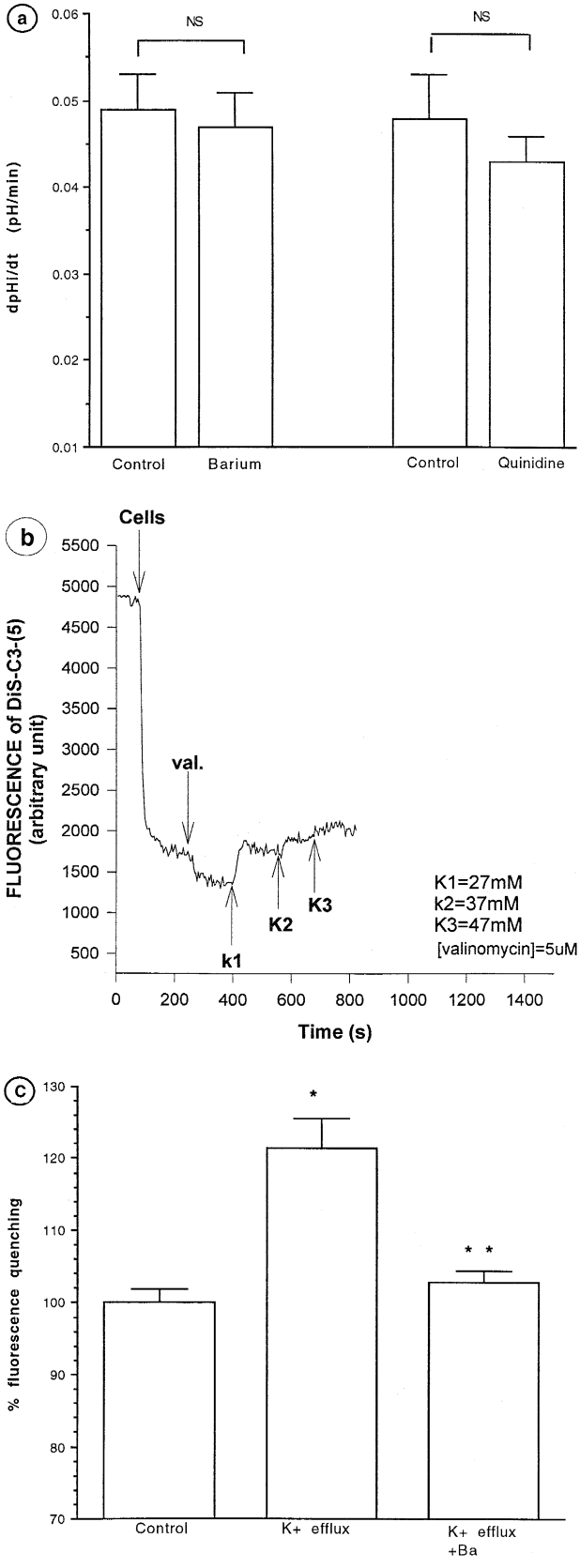


Fig. 5. Electroneutrality of K^+ efflux-induced NH_4^+ entry. a. mIMCD-3 cells were incubated in a Na^+-free high K^+ solution (solution C, Table 1) and then exposed to 10 mM NH_4Cl. Thereafter, and at a steady-state condition ($pH_i \sim 7.40$), cells were switched to a Na^+-free low K^+ solution that contained 10 mM $NH_4Cl \pm 10$ mM barium or ± 500 μM quinidine. Each datum represents mean ± S.E. for 5 separate experiments. b. mIMCD-3 cell suspensions were preincubated in a Na^+-free high K^+ solution and were added to a Na^+-free low K^+ solution that contained 2 μM of DiS-C3-(5). The fluorescence of the probe was quenched. Thereafter, valinomycin (val.) was added and K^+ concentration was incrementally increased to 27, 37, and 47 mM using aliquots from a 3 M KCl stock solution ((b) is a representative reproduction of 4 experiments). c. The quench of DiS-C3-(5) probe was determined after cells were added into the same media (high K^+; control, $n = 7$) or into a low K^+ medium in the absence (K^+ efflux, $n = 6$) or in the presence of 10 mM $BaCl_2$ (K^+ efflux + Ba, $n = 5$). * $P < 0.004$ compared to control; ** $P < 0.01$ compared to K^+ efflux + Ba.

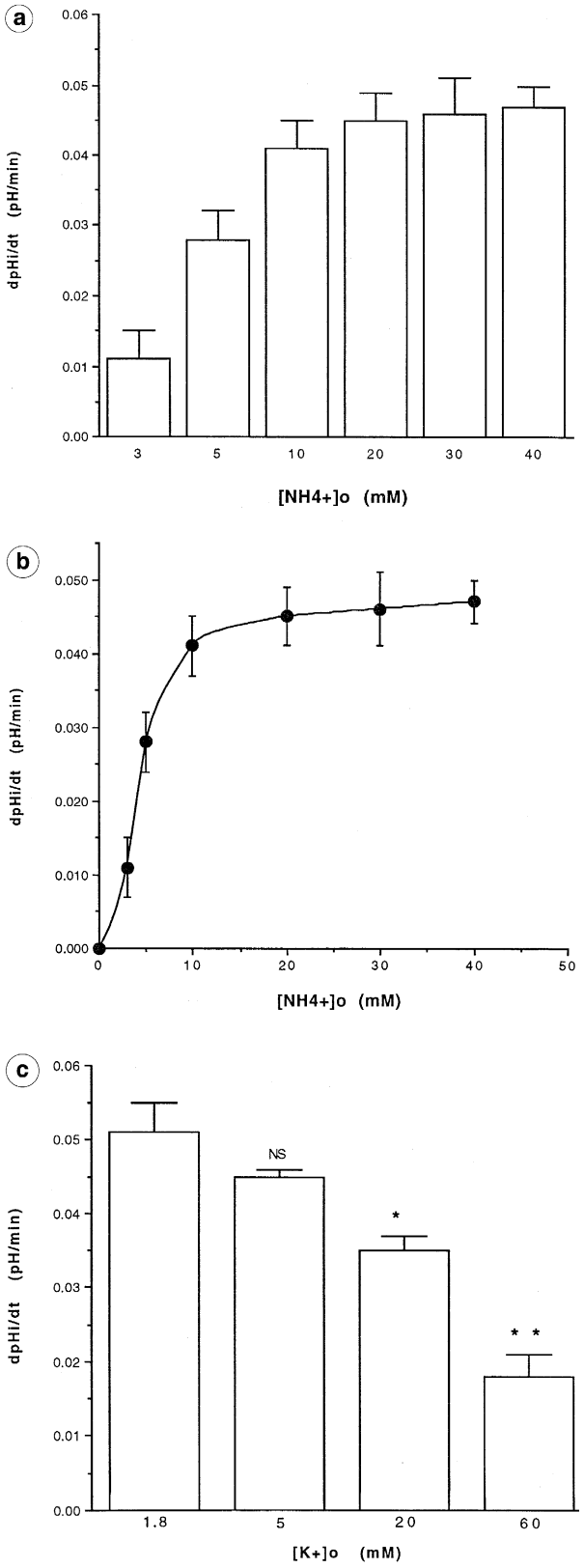
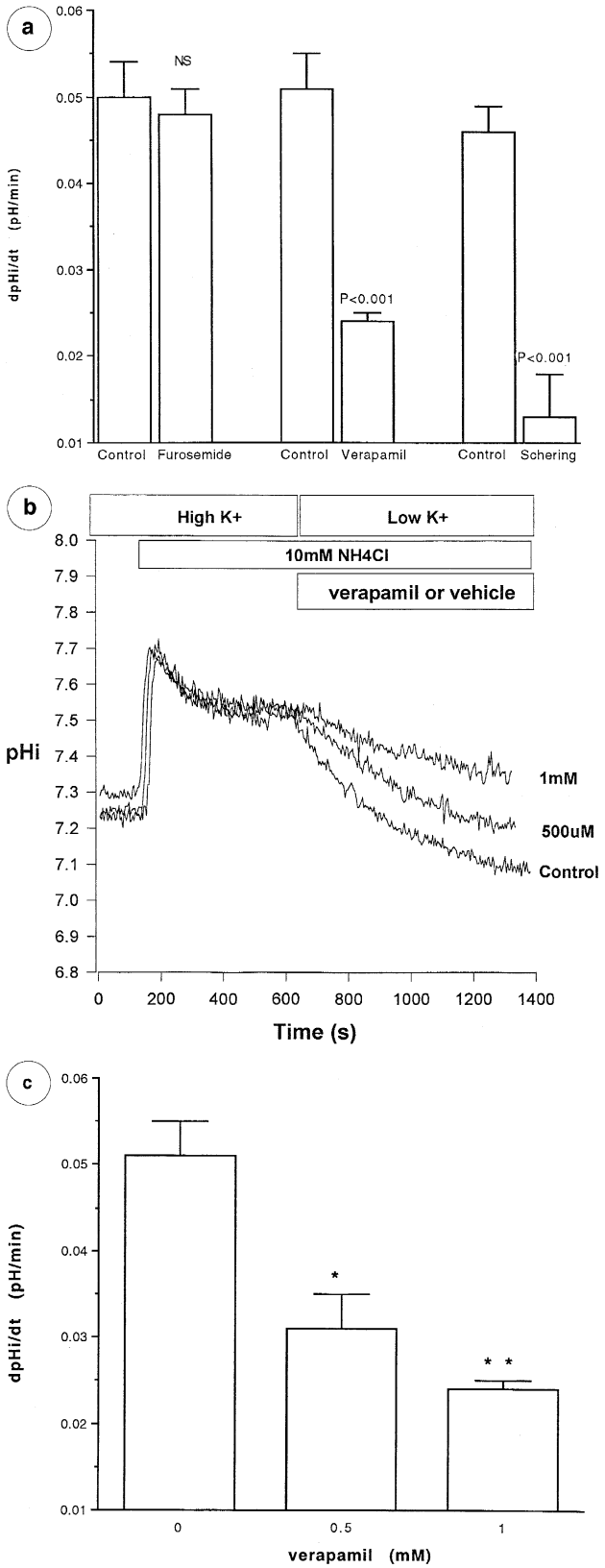


Fig. 6. Kinetic analysis of K^+/NH_4^+ antiport. Initial rate of K^+ efflux-induced NH_4^+ entry (dpH_i/dt) was measured in the presence of varying concentrations of NH_4^+ (a). Michaelis-Menten replot of the data shows a K_m of ~ 5 mM for NH_4^+ (b). Inhibitory concentration ($K_{1/2}$) of external K^+ on K^+/NH_4^+ antiport at constant NH_4^+ concentration is shown in (c). Each datum represents mean $\pm$ S.E. for 5 separate experiments; * $P <$ 0.003 compared to 1.8 mM K^+; ** $P < 0.001$ compared to 20 mM K^+.

entry into mIMCD-3 cells was significantly blocked in high K^+ solution. The results of five separate experiments (Fig. 3b) showed that the rate of NH_4-induced cell acidification was significantly lower in high K^+ media vs low K^+ media (0.049 ± 0.003 vs 0.018 ± 0.001 pH/min for 1.8 mM K^+ vs 120 mM K^+ in the media, respectively, $P < 0.001$). The baseline pH_i before and the peak pH_i following exposure to NH_4^+ were not different between the two groups.

3.4. K^+ efflux-induced NH_4^+ entry

The studies shown in Fig. 3 were performed in cells incubated in high or low K^+ solution for the entire duration of the experiments. To gain insight into the mechanism of interaction of K^+ with NH_4^+, the NH_4^+ transport into mIMCD-3 cells was studied upon imposition of an outward K^+ gradient. The purpose of this approach was to study the effect of K^+ efflux on NH_4^+ entry. Accordingly, cells were incubated in the presence of high K^+ (solution C, Table 1), exposed to ammonium (10 mM KCl was replaced with 10 mM NH_4Cl), and monitored for pH_i changes (the experiments were performed in the presence of 10 mM NH_4Cl, as K-dependent NH_4^+ entry is saturated at this concentration of NH_4^+, see Fig. 6 on kinetic analysis of this transporter). Following initial cell alkalinization and reaching steady-state pH_i, an outward K^+ gradient was imposed across the cells by changing to a low K^+-containing solution with 10 mM NH_4^+ (10 mM TMA-Cl of solution B was replaced with 10 mM NH_4Cl). As shown in Fig. 4a, imposing an outward K^+ gradient acutely acidified the cells. The results of five separate experiments showed that imposing an outward K^+ gradient resulted in 0.264 ± 0.032 pH units acidification in 10 min. This acidification was only observed in the



presence of NH_4^+, as imposing an outward K^+ gradient in the absence of NH_4^+ but in the presence of comparable alkaline pH_i (using acetate withdrawal method) did not induce any significant cell acidification (representative tracing in Fig. 4b).

3.5. Electroneutrality of K^+ efflux-induced NH_4^+ entry

Alterations in extracellular K^+ could affect the magnitude of membrane potential. Conceivably, acute NH_4^+-induced cell acidification that was observed in the presence of an outward K^+ gradient could be due to membrane hyperpolarization. To address this issue, the effect of two known K^+ channel inhibitors (barium and quinidine) on K^+ efflux-coupled NH_4^+ entry was examined. Fig. 5a demonstrates that addition of 10 mM barium or 500 μM quinidine to low K^+ solutions did not inhibit the NH_4^+-dependent K^+ efflux-induced cell acidification. To determine whether 10 mM barium could inhibit the cell membrane hyperpolarization induced by K^+ efflux, variations in membrane potential were measured by the use of DiS-C3-(5) probe as described in Section 2. Fig. 5b is a representative tracing demonstrating sensitivity of DiS-C3-(5) to membrane potential variation and shows that imposing an outward K^+ gradient causes significant quenching in DiS-C3-(5) fluorescence. Addition of valinomycin causes further quenching due to cell membrane hyperpolarization caused by further efflux of K^+ via the ionophore. Increasing the extracellular K^+ concentration gradually increased the fluorescence due to progressive membrane depolarization and, as a result, exit of the dye from the cells. These observation indicate the sensitivity of DiS-C3-(5) probe to membrane poten-

Fig. 7. Inhibition of K^+/NH_4^+ antiport by verapamil. mIMCD-3 cells were incubated in Na^+-free high K^+ medium and then exposed to 10 mM NH_4Cl. At steady-state pH_i, cells were perfused with Na^+-free low K^+ media in the absence or presence of 1.5 mM furosemide, 1 mM verapamil, or 300 μM Schering 28080 (a). b. Representative pH_i tracings of NH_4^+ transport inhibition at 0.5 and 1 mM verapamil. c. Each datum represents mean ± S.E. for control ($n = 12$), 0.5 mM verapamil ($n = 5$; * $P < 0.02$ compared to control), or 1 mM verapamil ($n = 5$; ** $P < 0.001$ compared to control).

tial variations. To determine whether barium blocked membrane hyperpolarization resulting from an outward imposition of K^+ gradient, the experiments were performed in the presence or absence of barium in the solution. As shown in Fig. 5c, imposing an outward K^+ gradient (middle bar) significantly increased quenching of the probe as compared to no outward K^+ gradient (left bar), consistent with membrane hyperpolarization. The presence of 10 mM barium completely blocked K^+-efflux induced membrane hyperpolarization (right bar). Presence or absence of NH_4^+ had no effect on membrane potential variation. Thus, the K^+ efflux-induced NH_4^+-dependent cell acidification occurred both with membrane hyperpolarisation (concomittant K^+ efflux through K^+ channel) and no change in the cell PD (K^+ channel blockade by barium). These results indicate that K^+-efflux induced NH_4^+ entry into mIMCD-3 cells is not mediated via alterations in membrane potential, and further, that this transporter is likely an electroneutral process. To study the properties of this transporter further, the rest of the experiments were performed using the K^+ efflux-coupled NH_4^+ entry protocol as described in Fig. 4a.

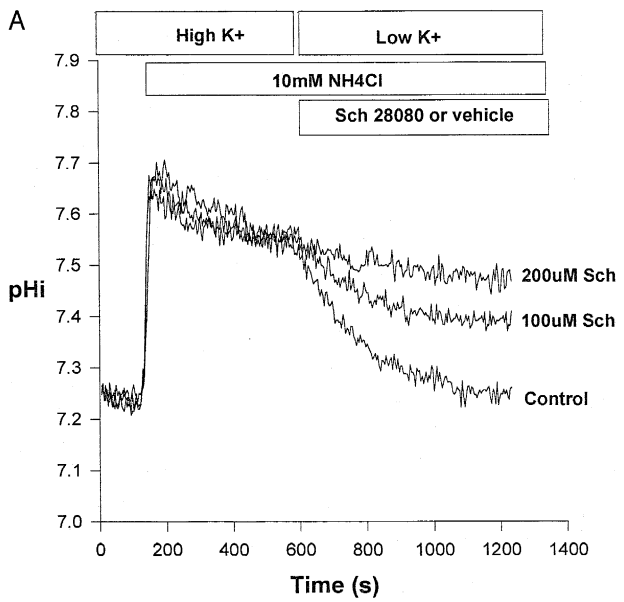


Fig. 8. Inhibition of K^+/NH_4^+ antiport by Schering 28080. mIMCD-3 cells incubated in Na^+-free high K^+ medium were exposed to 10 mM NH_4Cl, Following the initial cell alkalinization, the cells were perfused with Na^+-free low K^+ solution in the presence of 10 mM $NH_4Cl \pm$ Schering 28080. a. Representative pH_i tracings in the absence or in the presence of various concentrations of Sch28080. b. Each datum represents mean $\pm$ S.E. for 4–7 separate experiments. Inset: Dixon plot of data ($r = 0.994$).

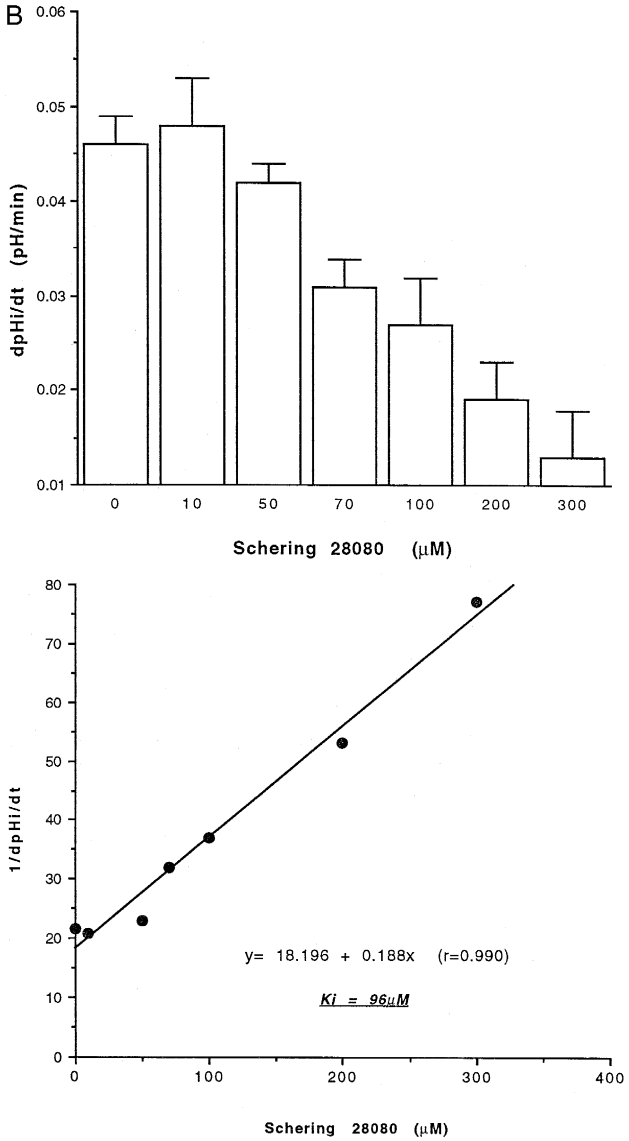


Fig. 8 (continued).

3.6. Kinetic analysis of K^+-NH_4^+ antiport

The results of the above experiments indicate that NH_4^+ transport in mIMCD-3 cells is independent of sodium, is coupled to K^+ efflux, is not mediated via K^+ channels, and is competitively inhibited by exter-

nal K^+. These results are consistent with an NH_4^+/K^+ antiport. Such a transporter could exchange extracellular NH_4^+ with intracellular K^+ and mediate the transport of NH_4^+ in mIMCD-3 cells. In the next series of experiments, the kinetics of the K^+/NH_4^+ antiporter were studied. To determine the relative affinity of NH_4^+ for the K^+/NH_4^+ antiporter, the rate of cell acidification was monitored in the presence of an increasing concentration of NH_4^+ at constant outward potassium gradient. NH_4^+-induced cell acidification was a saturable process with respect to NH_4^+ concentration (Fig. 6a): the K_m for NH_4^+ was ~ 5 mM (Fig. 6b).

The purpose of the next series of experiments was to study the interaction of external K^+ with NH_4^+ transport. Cells were incubated in high K^+ solution (solution C, Table 1) and were then exposed to 10 mM NH_4Cl and allowed to reach steady state pH_i. Thereafter, cells were switched to solutions with varying K^+ concentration (1.8 to 60 mM) at constant NH_4^+ concentration. As shown in Fig. 6c, external K^+ inhibits NH_4^+ transport in a concentration dependent manner.

3.7. Inhibitory profile of K^+/NH_4^+ antiport

K^+/NH_4^+ antiport was not inhibited by ouabain, amiloride, quinidine, bumetanide, and barium (Figs. 1 and 5 and Table 2). To examine the inhibitory profile of K^+/NH_4^+ antiport further, the effect of a variety of inhibitors was tested. Fig. 7a shows the effect of 1.5 mM furosemide, 1 mM verapamil, and 300 μM Schering 28080 on K-dependent NH_4^+ transport. The results indicated that two inhibitors amongst all chemicals tested, verapamil and Schering 28080, inhibited K^+/NH_4^+ antiport. Shown in Fig. 7b are pH_i tracings monitoring K^+ efflux-induced NH_4^+ entry in the presence of 0.5 and 1 mM verapamil compared to no inhibitor. The results of these tracings and five separate coverslips (Fig. 7c) indicate that verapamil significantly inhibited K^+/NH_4^+ antiporter. The inhibitory effect of Schering 28080 on K^+/NH_4^+ antiport was next tested. Fig. 8a illustrates tracings of Schering 28080-induced inhibition of NH_4^+-induced pH_i alterations in mIMCD-3 cells. Shown in Fig. 8b is dose response inhibition of the effect of Schering 28080 on dpH_i/dt. The Dixon plot of the dpH_i/dt values yielded a straight line consistent with a single Sch28080 site having an apparent inhibitory constant (K_i) of 96 μM (Fig. 8b, inset).

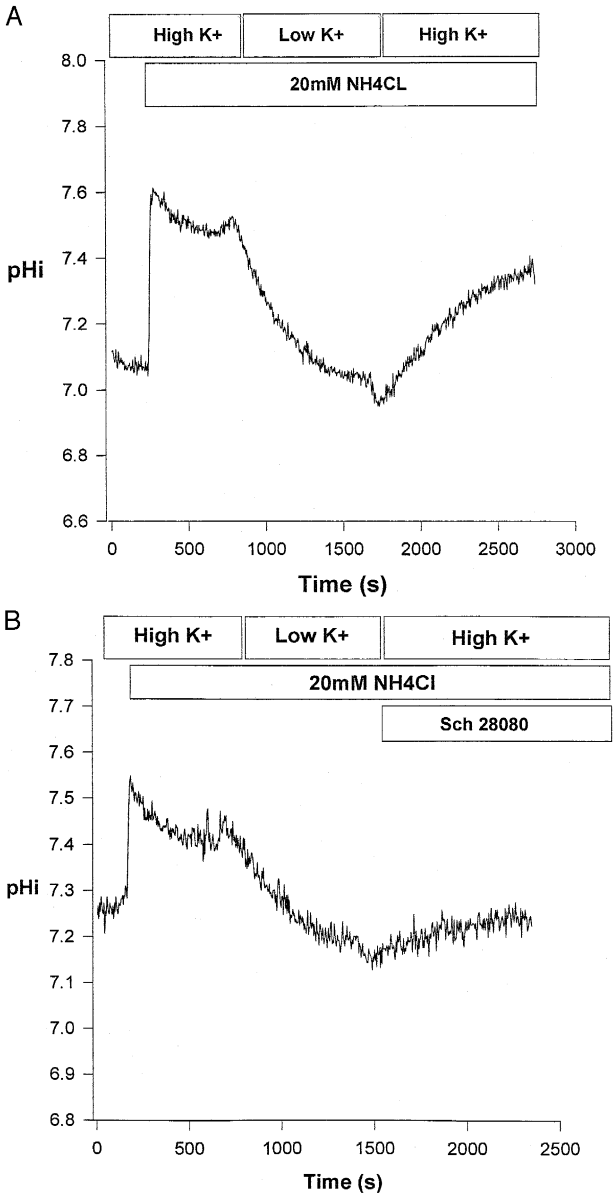


Fig. 9. Modes of K^+/NH_4^+ antiport function. mIMCD-3 cells loaded with NH_4^+ (20 mM NH_4Cl) were allowed to reach steady pH_i in low K^+ solution. Thereafter, cells were switched to a high K^+ solution in the presence of 20 mM NH_4Cl. a. Representative pH_i tracing showing cell alkalinization upon imposition of an inwardly directed K^+ gradient. b. Shows that high K^+-induced NH_4^+ efflux was abolished by 200 μM Sch28080.

3.8. Modes of K^+/NH_4^+ antiport operation

To determine whether K^+/NH_4^+ antiport can operate in a reverse mode, cells were incubated with NH_4^+ in the presence of an outward K^+ gradient for 15 min in a manner similar to previous figures. The cell acidification following the initial alkalinization was complete at 15 min as shown by pH_i tracing, indicating presence of equilibrium state and therefore NH_4^+ loading of the cells (Fig. 9a). The cells were then switched to a high K^+ solution (solution C). Exposure of NH_4^+-loaded cells to NH_4^+-containing high K^+ solution resulted in rapid cell alkalinization (Fig. 9a). The results of 4 separate experiments showed that switching NH_4^+-loaded cells from low (1.8 mM) to high (130 mM) K^+ increased the pH_i by 0.36 ± 0.09 pH unit, $P < 0.01$ (Fig. 9a). In the presence of Schering 28080, 200 μM, K^+-induced cell alkalinization was blocked (Fig. 9b). Switching mIMCD-3 cells from low to high K^+ solution in the absence of NH_4^+ had no effect on steady state pH_i ($n = 5$, data not shown). The results of the above experiments are consistent with K^+-induced NH_4^+ efflux, indicating that the K^+/NH_4^+ antiport can function in a reverse direction.

4. Discussion

NH_4^+ excretion is the major mechanism by which the body eliminates the increased metabolic acid load and therefore is essential for maintenance of acid base balance [1]. It has been shown that decreased NH_4^+ excretion impairs the ability of the body to eliminates metabolically-generated acid load and conversely, increased NH_4^+ excretion enables the body to get rid of the increased acid load [1,4–6]. Ammonium is synthesized in the mitochondria of proximal tubule cells by deamination of glutamine, transported to the cytosol and excreted in part via the Na^+/H^+ exchanger [7,8]. Once in the lumen of proximal tubule, it is delivered to medullary thick ascending limb of Henle where it is reabsorbed via a number of transport pathways [9–12,17]. The reabsorbed NH_4^+ enters the interstitium, is transported to the medullary collecting duct cells, and secreted into the collecting duct lumen [1,4–6]. The mechanism of NH_4^+ transport in the medullary collecting duct is poorly understood [1].

Present studies suggest that NH_4^+ entry in mouse cultured inner medullary collecting duct (mIMCD-3) cells is carrier-mediated. NH_4^+ entry occurs via a K^+-dependent pathway that was distinct from Na^+-K^+-ATPase, Na^+-K^+-$2Cl^-$, K^+/H^+ antiport, or K^+-channel (Figs. 1 and 2 and Table 2). Imposing an outward K^+ gradient (K^+ efflux) was coupled to enhanced NH_4^+ entry into mIMCD-3 cells (Fig. 4). Coupling of K^+ efflux to NH_4^+ entry was not affected by alterations in membrane potential (Fig. 5). Indeed, 10 mM barium or 500 μM quinidine which blocks potassium channels had no effect on NH_4^+ transport (Fig. 5). The results further demonstrated that NH_4^+ transport was not driven by secondary alterations in membrane potential, but rather was mediated by an electroneutral transport process (Fig. 5c). Taken together, these results are consistent with the presence of K^+/NH_4^+ antiport in mIMCD-3 cells. The K^+/NH_4^+ antiporter did not show any affinity for H^+ (Fig. 4), thus, making it distinct from the $K^+/H^+/NH_4^+$ transporter [18].

The results of the experiments in Figs. 1–3 show that absence of sodium or presence of bumetanide did not affect NH_4^+ transport, indicating that Na^+:K^+:$2Cl^-$ dose not contribute to NH_4^+ transport in mIMCD-3 cells. These results are interesting since studies in mTAL cells have shown that NH_4^+ absorption occurs predominantly via the Na^+:K^+:$2Cl^-$ cotransporter [9–12]. While mIMCD-3 cells express a Na^+:K^+:$2Cl^-$ cotransporter, the structure of that gene [27] is distinct from the luminal mTAL Na^+:K^+:$2Cl^-$ cotransporter [28]. Moreover, the basolateral membrane localization of Na^+:K^+:$2Cl^-$ cotransporter in mIMCD − 3 cells [27] suggest that these two isoforms perform different functions. Amiloride, an inhibitor of Na^+/H^+ exchange or Na^+ channel [29], did not block NH_4^+ entry, indicating lack of involvement of these two transporters in mIMCD-3 cells. Recent studies in kidney proximal tubule have shown that Na^+/H^+ exchange transports NH_4^+ [7]. Lack of involvement of Na^+/H^+ in mediating NH_4^+ transport in mIMCD-3 cells may reflect either isoform difference (NHE-2 in mIMCD-3 cells vs NHE-3 in proximal tubule cells) or tissue specificifty in this regard.

The role of Na^+:K^+-ATPase in NH_4^+ transport has been controversial. Several recent reports have shown

no affinity of Na^+:K^+-ATPase for NH_4^+ in kidney tubules and vascular smooth muscle cells [15,30,31]. However, two studies in microperfused rabbit proximal tubule [14] and rat medullary collecting duct [32] have shown that Na^+:K^+-ATPase can interact with NH_4^+. The interaction of NH_4^+ with Na^+:K^+-ATPase in the latter studies [32] was demonstrated by inhibition by ouabain of NH_4Cl-induced increase in net acid secretion in rat IMCD at steady state. The results of those experiments are not in conflict with the current experiments. In the present experiments, we found that Na^+:K^+-ATPase did not contribute to NH_4^+ transport in mIMCD-3 cells, as measured by initial entry of NH_4^+ into mIMCD-3 cells. However, the contribution of Na^+:K^+-ATPase to the initial rate of NH_4^+ transport was not studied in rat IMCD microperfusion experiments [32]. It is therefore likely that inhibition of Na^+:K^+-ATPase by ouabain, as employed in steady state net acid secretion measurements [32], could have depleted intracellular $[K^+]$ and thus inhibited K^+/NH_4^+ antiporter. In support of this view, we find that depletion of intracellular K^+ by preincubation of mIMCD-3 cells in K^+ and Na^+-free solution for 30 minutes inhibited NH_4^+ entry into mIMCD-3 cells (0.053 ± 0.004 in normal K vs 0.039 ± 0.002 pH/min in K^+-depleted cells, $P < 0.02$). Another plausible explanation in this regard could be species specificity of NH_4^+ affinity for Na^+:K^+-ATPase as those studies were performed in rat and rabbit [13,14,32]. Our results are consistent with the results of studies in kidney proximal tubule cells [15], cortical collecting duct cells [31], and vascular smooth muscle cells [30] indicating that Na^+-K^+-ATPase is not involved in NH_4^+ transport.

The K^+/NH_4^+ antiport that is described in the present studies is distinct from the K^+/H^+ antiport in rat mTAL cells that carries NH_4^+ [18] or K^+/H^+ antiport that is identified in cultured opposum kidney (OK) cells [33]. The K^+/NH_4^+ antiport has no affinity for H^+ as shown in Fig. 4b demonstrating that in the absence of NH_4^+, induction of an outward K^+ gradient had no effect on cell pH. We further find that in the absence of NH_4^+, increasing the extracellular potassium (from 1.8 to 140 mM mM) had no effect on pH_i (data not shown). In addition, the K^+/NH_4^+ antiporter was not inhibited by barium (Fig. 1c, Fig. 5a and Table 2). Taken together, these results indicate that the K^+/NH_4^+ antiporter is distinct from K^+/H^+ antiport in mTAL cells (which has affinity for NH_4^+) or from K^+/H^+ antiport described in (OK) cells [18,33]. The K^+/NH_4^+ antiport is also distinct from the H^+/K^+-ATPase described in various nephron segments [34–37]. While we have not examined the affinity of NH_4^+ for the K^+ binding site of H^+/K^+-ATPase, the results do not support a role for H^+/K^+-ATPase in NH_4^+ transport in mIMCD-3 cells for the following reasons. First, a possible exchange of extracellular NH_4^+ for intracellular H^+ via H^+/K^+-ATPase should at best be neutral with respect to pH_i. However, the transport of NH_4^+ in the current experiments (Figs. 1–9) causes cell acidification, indicating the exchange of NH_4^+ with a non-acidic cation (i.e. K^+ in this occasion). Moreover, the sensitivity of the K^+/NH_4^+ antiport inhibition to Schering 28080 (IC50 ~ 96 μM) is much less than the sensitivity of the H^+/K^+-ATPase to this inhibitor (IC50 < 10 μM) reported in mIMCD-3 cells [37] and other investigations [34]. Lastly, the K^+-induced cell alkalinization in mIMCD-3 cells loaded with NH_4^+ (Fig. 9) indicates that this transporter exchanges K^+ for NH_4^+ and could function in a reverse mode, a feature not shared by H^+/K^+-ATPase.

The K^+/Cl^- cotransporter has been shown to accept NH_4^+ in place of K^+ in mTAL cells of rat kidney [12]. However, the NH_4^+ transport in mIMCD-3 cells is not mediated via K^+/Cl^- cotransport, as barium (Fig. 5a) and furosemide (Fig. 7a), two strong inhibitors of K^+/Cl^- cotransport [12,38–40], had no effect on NH_4^+ dependent cell acidification in mIMCD-3 cells. An amiloride-sensitive NH_4^+ conductance and a nonspecific amiloride-sensitive cation channel in mTAL [18] and IMCD [41] cells have shown affinity for NH_4^+. However, the presence of amiloride had no effect on NH_4^+ transport in mIMCD-3 cells, indicating lack of involvement of this channel (Table 2).

The kinetic analysis studies (Fig. 6c) demonstrate that increasing the K^+ concentration from 5 to 20 mM significantly inhibited NH_4^+ entrance in mIMCD-3 cells. These are relevant physiological concentrations with respect to K^+ concentration in the interstitium of inner medulla [35,42] and indicate that increasing or decreasing the K^+ concentration within the physiologic range could significantly affect NH_4^+ entrance in mIMCD-3 cells and, as a

result, regulate acid secretion into the urine. If such were the case, hyperkalemic metabolic acidosis [43,44] may in part result from inhibition of NH_4^+ entrance into the medullary collecting duct cells. Conversely, potassium depletion metabolic alkalosis [43,45,46] may, in part, result from enhanced NH_4^+ entrance into the medullary collecting duct cells. The affinity of NH_4^+ for NH_4^+-binding site of K^+/NH_4^+ antiport was found to be ~ 5 mM (Fig. 6); NH_4^+ concentration in the inner medulla is about ~ 10 mM [5,6]. Thus, alterations in NH_4^+ concentration within physiologic range could significantly affect the activity of K^+/NH_4^+ antiporter and change the rate of NH_4^+ entrance in mIMCD-3 cells. This in turn would alter the rate of luminal H^+ secretion and, as a result, HCO_3^- reabsorption.

The K^+/NH_4^+ antiporter has so far been observed only in mIMCD-3 cells and not in proximal tubule cells [15] or other nephron segments. Thus, this transporter may be unique to the inner medulla. Given the K^+ and NH_4^+ concentration gradients in the interstitium and the lumen of the inner medullary collecting duct cells and the non-ionic diffusion of NH_3 at the luminal membrane, we suggest that this transporter is located in the basolateral membrane of inner medullary collecting duct cells and is likely responsible for the transport of NH_4^+ from the interstitium to the cells of this nephron segment. Such a localization of K^+/NH_4^+ antiporter in IMCD cells would provide, physiologically, an efficient mean of NH_4^+ transport as well as K^+ recycling in the inner medulla. Moreover, NH_4^+ entrance via K^+/NH_4^+ antiporter may also serve as a proton source for luminal H^+ secretion and bicarbonate absorption. The latter possibility is suggested by studies in perfused cortical collecting tubules showing that total proton flux is greater in the presence of NH_4^+ than its absence [47]. The inhibition of K^+/NH_4^+ antiporter by verapamil which inhibits K^+ channel [48] and by Sch-28080 which competes with the K^+ binding site of the gastric H^+-K^+-ATPase [49] suggests that these agents may interact with the K^+-(or NH_4^+) binding site of the K^+/NH_4^+ antiporter. Finally, the K^+/NH_4^+ antiporter is likely driven by an outwardly directed K^+ concentration gradient generated and maintained by Na^+-K^+ ATPase activity. Equilibrium thermodynamics predict that the K^+/NH_4^+ antiporter will be at equilibrium and will mediate no net flux when $[K^+]_i/[K^+]_o$ equals $[NH_4^+]_o/[NH_4^+]_i$. To determine the exact stoichiometry of K^+/NH_4^+ antiport (i.e. whether it is $1K^+$ per $1NH_4^+$ or whether there is a modifier site) one needs to measure intracellular K^+ and NH_4^+ concentrations in the presence of various outward K^+ or inward NH_4^+ gradients.

In conclusion, a new transporter, called K^+/NH_4^+ antiporter, has been described in inner medullary collecting duct cells. This transporter exchanges intracellular K^+ with extracellular NH_4^+, is inhibited by verapamil and Schering 28080, and is affected by physiologic alterations in K^+ or NH_4^+ concentrations. The K^+/NH_4^+ antiporter may play an essential role in regulation of acid base balance.

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